KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
LAMP1	Cell Signaling Technologies	cat# 9091
Phospho-S6 Ser240/244	Cell Signaling Technologies	cat# 2215
S6	Cell Signaling Technologies	cat# 2217
PAK1	Cell Signaling Technologies	cat# 2602
Phospho-p70S6K Thr389	Cell Signaling Technologies	cat# 9234
p70S6K	Cell Signaling Technologies	cat# 9202
Tubulin	Sigma-Aldrich	cat# T4026
PTEN	Cell Signaling Technologies	cat# 9188
ATG5	Cell Signaling Technologies	cat# 8540
LC3	Cell Signaling Technologies	cat# 4108
AMPK	Cell Signaling Technologies	cat# 2532
Phospho-AMPK Thr 172	Cell Signaling Technologies	cat# 2535
Phospho-ACC site Ser79	Cell Signaling Technologies	cat# 3661
ACC	Cell Signaling Technologies	cat# 3676
TetR Monoclonal Antibody (Clone 9G9)	Clontech	cat# 631131
IRDye® 800CW Goat anti-Rabbit IgG (H + L) 0.5 mg	Licor	P/N 926-32211
IRDye® 680RD Goat anti-Mouse IgG (H + L) 0.5 mg	Licor	P/N 926-68070
Media and additives		
DMEM With L-Glutamine; with 4.5g/L Glucose; without Sodium; Pyruvate	Fisher	MT10017CV
RPMI 1640	Fisher	MT10040CM
RPMI 1640 (ATCC modification)	Life Technologies	A1049101
Keratinocyte-SFM (1X)	Life Technologies	17005042
F-12K with Glutamine	Fisher	MT10025CV
EGF Recombinant Human Protein	Life Technologies	PHG0311
Bovine Pituitary Extract	Life Technologies	13028014
Insulin Solution From Bovine Pancreas	Sigma-Aldrich	I0516-5mL
HEPES buffer	Fisher	25-060-CI
L-Glutamine	Fisher	MT25005CI

β-mercaptoethanol	Sigma-Aldrich	M-6250
100X MEM Vitamins	Life Technologies	11120052
100X RPMI Vitamins	Sigma-Aldrich	R7256-100ML
Human prostate organoid medium	(Gao et al., 2014)	N/A
Mouse prostate organoid medium	(Chua et al., 2014)	N/A
SILAC amino acids: Arg10-HCL	Silantes	201604102
SILAC amino acids: Lys-4d:2HCL	Silantes	211104113
FBS SILAC	Thermo Fisher Scientific	88440
RPMI 1640 SILAC	Life Technologies	A2494401
Lys-C Endoproteinase	Promega	V1071
Trypsin	Sigma	T4799
Chemicals, Peptides, and Recombinant Proteins		
AnnexinV-FITC	VWR	640905-BL
CellTrace CFSE Cell Proliferation Kit	Life Technologies	C34554
DAPI	VWR	422801-BL
Hoechst 33342, trihydrochloride, trihydrate	Life Technologies	H3570
Oregon Green dextran	Life Technologies	cat# D7173
Texas Red dextran	Life Technologies	cat# D1864
DQ BSA	Life Technologies	cat# D12050
FITC-Ficoll	Sigma-Aldrich	cat# 51731
Lysotracker Red	Life Technologies	cat# L-7528
Lysotracker Blue	Life Technologies	cat# L-7525
EIPA	Cayman Chemical	cat# 14406
EHT1864	Cayman Chemical	cat# 17258
AZD8186	Selleckchem	Cat# S7694
BYL719	Selleckchem	cat# S2814
bpV(HOpic)	Sigma-Aldrich	SML0884
Compound C	Sigma-Aldrich	cat# P5499
A769662	Selleckchem	cat# s2697
Rapamycin	VWR	80054-244
Blasticidin S Hydrochloride	VWR	89149-988
Hygromycin B	Sigma-Aldrich	H3274-250MG
Doxycycline Hydrochloride	Calbiochem	324385
Puromycin Dihydrochloride	VWR	89158-882
Fatty acid free BSA	Sigma-Aldrich	cat# A8806
0.25% Trypsin, 0.1% EDTA in HBSS w/o Calcium, Magnesium and Sodium Bicarbonate	Fisher	MT25053CI
Critical Commercial Assays		
Gibson Assembly® cloning kit (NEB)	New England Biolabs	102500-052
Deposited Data		

Experimental Models: Cell Lines		
PTEN WT MEF	Edinger lab (C57BL/6 background, p53 null)	(Kim et al., 2016)
PTEN KO MEF	Edinger lab (C57BL/6 background, p53 null)	(Kim et al., 2016)
AMPK WT MEF	P53 null, from Benoit Viollet via Reuben Shaw (Salk Institute)	(Laderoute et al., 2006)
AMPK DKO MEF	P53 null, from Benoit Viollet via Reuben Shaw (Salk Institute)	(Laderoute et al., 2006)
PAK1 WT MEF	Jonathan Chernoff (Fox Chase Cancer Center)	
PAK1 KO MEF	Jonathan Chernoff (Fox Chase Cancer Center)	
ATG5 WT MEF	Noboru Mizushima (University of Tokyo)	
ATG5 KO MEF	Noboru Mizushima (University of Tokyo)	
LKB1 WT	Reuben Shaw (Salk Institute)	(Shaw et al., 2004)
LKB1 KO	Reuben Shaw (Salk Institute)	(Shaw et al., 2004)
mPCE	Edinger lab (from a prostate tumor in a *Pten*$^{flox/flox}$;*p53*$^{flox/flox}$; *PB*-Cre4 male mouse))	(Kim et al., 2016)
PC3	ATCC	CRL-1435
DU145	ATCC	HTB-81
LNCaP	ATCC	CRL-1740
RWPE-1	ATCC	CRL-11609
FL5.12	Edinger lab	(Dexter et al., 1980)
LSL MEF	David Tuveson (Cold Spring Harbor Laboratory)	(Tuveson et al., 2004)
KRASG12D MEF	David Tuveson (Cold Spring Harbor Laboratory)	(Tuveson et al., 2004)

PTEN WT prostate organoid	Edinger lab (from C57BL/6 mouse prostate)	N/A
MSKPCa1 organoid	Yu Chen (MSKCC), provided by Dong Gao	(Gao et al., 2014)
Experimental Models: Organisms/Strains		
C57BL/6 mice	Jackson Laboratory	Stock# 000664
NSG mice bearing CRPC PDX TM00298	Jackson Laboratory	
pDKO mice on the C57BL/6 background generated using *Pten*$^{flox/flox}$ mice, *p53*$^{flox/flox}$ mice, and *PB-Cre4* mice	In vitro fertilization performed by the Transgenic Mouse Facility at UC Irvine using female *Pten*$^{flox/floxx}$;*p53*$^{flox/flox}$ and male *Pten*$^{flox/flox}$;*p53*$^{flox/flox}$; *PB-Cre4* mice bred in Edinger lab	(Kim et al., 2016)
Pten$^{flox/flox}$ mice	Jackson Laboratory	No. 0045597
p53$^{flox/flox}$ mice	Jackson Laboratory	No. 008462
PB-Cre4 mice	NCI-Frederick Mouse Repository	strain # 01XF5
Recombinant DNA		
pMIGR AMPK KD	Addgene (deposited by Morris Birnbaum)	Plasmid # 27296
CyPet-RAC1 (dTurquoise-RAC1)	Digman lab (gift from Klaus Hahn)	(Hinde et al., 2012) www.hahnlab.com
pTriex YPet-PBD p2a t2a dTurquoise RAC1 (RAC1 FLARE dual-chain biosensor)	Digman lab (gift from Klaus Hahn)	(Hinde et al., 2012) www.hahnlab.com
CyPet-RAC1-Q61L	Digman lab (gift from Klaus Hahn)	(Hinde et al., 2012) www.hahnlab.com
YPet-PBD	Digman lab (gift from Klaus Hahn)	(Hinde et al., 2012) www.hahnlab.com
1066 pBabe puroL PTEN	Addgene (deposited by William Sellers)	Plasmid# 10785
pBabe-puro	Addgene (deposited by Hartmut Land & Jay Morgenstern & Bob Weinberg)	Plasmid# 1764
pMA2640	Addgene (deposited by Mikhail Alexeyev)	Plasmid# 25434
pRK5-Myc-RAC1-T17N	Addgene (deposited by Gary Bokoch)	Plasmid #12984
pRevTRE	Clontech	Cat#631002
Sequence-Based Reagents		
Px330 PTEN CRISPR	Addgene	Plasmid # 59909
pRevTRE Fwd: GCATGCAAGCTTGTTAACATCG	Edinger Lab	N/A

PTEN-pRevTRE Fwd: GGATCCTCTAGAGTCGACCTGCGCCGCCATGACAGCCATCATCAAAGAGATC	Edinger Lab	N/A
PTEN-pRevTRE Rev: CGATGTTAACAAGCTTGCATGCTCAGACTTTTGTAATTTGTGTATGCTGATC	Edinger Lab	N/A
pRevTRE Rev: GCAGGTCGACTCTAGAGGATCC	Edinger Lab	N/A
Software and Algorithms		
LI-COR Odyssey	LI-COR	N/A
ImageJ	Freeware	N/A
GraphPad PRISM	GraphPad	N/A
Maxquant software 1.5.0.8	Freeware	N/A
SimFCS FLIM analysis software	Laboratory for Fluorescence Dynamics	http://www.lfd.uci.edu/
R	The R Foundation	https://www.r-project.org/
Other		
Mass spectrometer: Thermo Q-exactive HF	Thermo Fisher Scientific	IQLAAEGAAPFALGMBFZ

Contact for Reagent and Resource Sharing

Further information and requests for reagents may be directed to and will be fulfilled by the corresponding author, Aimee Edinger (aedinger@uci.edu).

Experimental Model and Subject Details

Cell culture. mPCE cells generated from prostate tumor tissue from a *Pten*$^{flox/flox}$;*p53*$^{flox/flox}$;*PB-Cre4* mouse were maintained in DMEM with 10% fetal bovine serum (FBS), 25 µg/ml bovine pituitary extract, 5 µg/ml bovine insulin, and 6 ng/ml recombinant human epidermal growth factor (mPCE media). FL5.12 cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 10 mM HEPES, 55 µM β-mercaptoethanol, antibiotics, 2 mM L-glutamine, and 500 pg/ml rIL-3. DU145 and MEF cells were cultured in DMEM with 4.5 g/L glucose and L-glutamine. PC3 cells were cultured in Ham's F12K media. RWPE-1 cells were cultured in Keratinocyte-SFM (Life Technologies) and LNCaP cells were cultured in RPMI medium – ATCC

modification (Life Technologies). All media (except RWPE-1 medium) were supplemented with 10% FCS and antibiotics. All commercial cell lines were obtained from ATCC (American Type Culture Collection) unless stated otherwise. MEFs and prostate cancer cell lines were passaged for ≤ 3 wk before low-passage vials were re-thawed. All prostate cancer cell lines were analyzed for loss of PTEN expression (western blotting) and AR expression (immunofluorescence). *Mycoplasma* testing was performed using the VENOR GeM PCR kit, (Sigma) every four months for all cell lines.

Dox-inducible system. For doxycycline-inducible PTEN expression, mPCE cells were transduced using standard protocols with pMA2640 to confer expression of the rtTA transcription factor and selected using 7.5 µg/ml Blasticidin S Hydrochloride. Clones were generated and screened by western blot for rtTA expression; a clone expressing moderate levels of rtTA was selected. The wild type PTEN cDNA was cloned from pBabe puroL PTEN into pRevTRE using the Gibson Assembly® cloning kit (NEB). pRevTRE-PTEN or pRevTRE empty vector was used to generate virus, and transduced rtTA-mPCE cells selected using 900 µg/ml of Hygromycin B (Sigma-Aldrich). Using the transduced population, doxycycline hydrochloride (Calbiochem) concentration was titrated to obtain a PTEN expression level similar to PTEN WT MEFs using western blotting. In studies using rtTA-mPCE-PTEN cells, PTEN expression was induced using 250 ng/ml of doxycycline 18 h prior to the experiment. Multiple clones were evaluated with similar results.

Organoid culture systems. To generate normal mouse prostate organoid, prostate from a 14 week old wild type C57BL/6 male mouse was resected and digested with collagenase following the protocol described in (Chua et al., 2014). Cells isolated from the prostate were seeded in organoid media with 5% Matrigel and allowed to grow for 4 weeks as described in (Chua et al.,

2014). mPCE spheroids were generated by seeding 5,000 mPCE cells in 5% Matrigel-containing mPCE media. MSK-PCa1 organoids were maintained in human prostate organoid media formulated as in (Gao et al., 2014).

Mouse Maintenance. Mice were maintained on autoclaved Envigo Teklab corncob (1/8th inch) under SPF conditions. Mice were housed in Techniplast individual ventilated cages (IVC) ≤ 5 mice per cage on ventilated racks at 21 ± 1°C on a 12 h light/dark cycle. Animals were fed Envigo Teklad 2020X global soy protein-free extruded rodent diet upon weaning at 21 days of age. Breeding was conducted in pairs or trios, and breeding mice were supplied with EnvigoTeklad 2019 global 19% protein extruded rodent diet.

To generate pDKO mice on the C57BL/6 background, *Pten*$^{flox/flox}$ mice (stock No. 0045597) and *p53*$^{flox/flox}$ mice (stock No. 008462) were obtained from the Jackson Laboratory and *PB-Cre4* mice (strain #01XF5) were obtained from the NCI-Frederick Mouse Repository. Age-matched cohorts of pDKO males were generated by *in vitro* fertilization executed with the assistance of the Transgenic Mouse Facility at UC Irvine. NOD SCID IL-2 receptor gamma deficient (NSG) mice bearing CRPC PDX TM00298 were purchased from the Jackson Laboratory.

Methods Details

Flow cytometry assays. Cell viability was measured by vital dye exclusion using either propidium iodide or DAPI at 1 μg/ml using an BD LSRII or FACS Calibur flow cytometer. Cell proliferation was determined by recording the number of cells that excluded vital dye (live cell count) over a fixed time interval (30 – 60 seconds). Proliferation is expressed as fold change based on a sample collected at T = 0 h. All flow assays were performed in triplicate (technical replicates) and the means from ≥ 3 independent experiments (biological replicates) averaged.

Light Microscopy. Bright-field and epifluorescence microscopy were conducted using a Nikon TE2000-S fluorescence microscope. Confocal microscopy was performed on a Nikon Eclipse Ti spinning disk confocal microscope. Where cells were nutrient deprived, dextran or BSA uptake was measured after a 16 h incubation in low nutrient medium. When the macropinocytosis inhibitor 5-(N-ethyl-N-isopropyl) amiloride (EIPA, 50-75 μM) was used, it was added during the final hour of incubation prior to dextran addition. Oregon Green dextran (1 mg/mL) or DQ-BSA (0.3 mg/ml) or Alexa 488 BSA (0.5 mg/ml) in PBS and Lysotracker Red (1:10,000 dilution) and Hoechst 33342 were added for 30 min, cells were washed three times with PBS, and live cells evaluated on the spinning disc confocal microscope. Macropinocytic index was calculated using ImageJ software as detailed in (Commisso et al., 2014). For Dil-LDL uptake, mPCE cells were incubated in media with 10% charcoal-stripped serum for 24 h then incubated with 20 μg/ml Dil-LDL +/- EIPA for 2 h.

Western Blotting. Cells were placed on ice, washed with ice-cold PBS, and lysed with ice-cold RIPA lysis buffer [150 mM NaCl, 50 mM Tris, pH 7.3, 0.25 mM EDTA, 1% (w/v) sodium deoxycholate, 1% (w/v) Triton X-100, 1 mM Na orthovandanate, 50 mM NaF, 10 mM Na pyrophosphate, 10 mM Na glycerophosphate, 1x Complete protease inhibitor (Roche)] and the lysate scraped off the plate using a cell scraper. Insoluble material was removed from lysates by centrifugation at 13,000 rpm at 4°C for 10 min. Protein in cell lysates was quantified using the Pierce BCA Protein Assay (Thermo Scientific). Equal amounts of protein were loaded on 4-12% NuPAGE gels (ThermoFisher) and transferred to nitrocellulose membranes. Membranes were blocked with 5% BSA in TBST (1x TBS, 0.01% w/v Tween-20) and exposed to primary antibodies overnight at 4°C rocking. Secondary antibodies coupled to infrared dyes were obtained from LI-COR. Analysis and quantification of blots was performed on a LI-COR® Odyssey® Sa imaging system.

Necrotic cell uptake assays. CFSE-labeled FL5.12 uptake was measured after prostate cancer cells were incubated for 16 h in low nutrient medium. To label FL5.12 cells with CFSE, live cells were spun down, washed once with PBS, then re-suspended at 500,000 cells/ml in PBS containing 1% FBS, IL-3, and 5 μM CFSE for 20-30 minutes. The CFSE-labeled FL5.12 cells were then spun down and the excess CFSE was quenched with media containing serum. CFSE-labeled necrotic FL5.12 cells were generated by washing labeled cells three times with PBS and then placing them in FL5.12 media without IL-3 at a density of 100 million cells/ml for 48 h. Apoptotic FL5.12 cells were generated by washing CFSE-labeled cells three times with PBS and placing them in FL5.12 media without IL-3 at a density of 25,000 cells/ml for 24 h. Live CFSE-labeled FL5.12 cells were re-suspended in FL5.12 media with IL-3 at a density of 500,000 cells/ml. One million necrotic or apoptotic cell equivalents were spun down at 13,000 rpm at 4°C for 10 min and fed to prostate cancer cells for 1 h prior to imaging (0.05% protein). Live CFSE-labeled FL5.12 cells were spun down at RT at 1,500 rpm for 3 min and fed to prostate cancer cells for 1 h prior to imaging. Lysotracker Red and Hoechst 33342 were added for the last 20 min of incubation. When the macropinocytosis inhibitor 5-(N-ethyl-N-isopropyl) amiloride (EIPA, 50-75 μM) was used, it was added during the final hour of incubation prior to FL5.12 addition. Prior to imaging, prostate cancer cells were washed 5 times with PBS then evaluated live on the spinning disc confocal microscope. The same CFSE labeled FL5.12 cells were also imaged on the Nikon TE2000-S fluorescence microscope with a 100x objective. Non-CFSE labeled necrotic, apoptotic, and live FL5.12 cells were prepared and stained with DAPI (1 μg/ml) and Annexin V-FITC (1:1000 v/v) for 10 min at RT in 1x Annexin V binding buffer (10 mM HEPES pH 7.4, 2.5 mM $CaCl_2$, 140 mM NaCl) at a concentration of 1 million cells or cell equivalents/ml. One million necrotic or apoptotic cell equivalents were spun down at 13,000 rpm at 4°C for 10 min. One million live cells were spun down at 1,500 rpm at RT for 3 min. FL5.12 cells were then imaged on a Nikon TE2000-S fluorescence microscope using the 40x objective.

Stable isotope labeling with amino acids in cell culture (SILAC). FL5.12 cells were incubated in SILAC media containing "heavy" ^{13}C- or ^{15}N-labeled arginine (R^{10}) and lysine (K^{4}) for 10 divisions. FL5.12 cells were killed by IL-3 withdrawal and debris from 100 million FL5.12 cells were pelleted and fed to prostate cancer cells. After 4-6 doublings, prostate cells were washed and harvested lysates were digested with Trypsin or Lys-C.

Mass Spectrometry Analysis. mPCE and DU145 cells were lysed using 1% sodium deoxycholate (SDC) in 50 mM ammonium bicarbonate after three washes each using 1 ml PBS. Proteins were quantified using Pierce BCA assay. Fifty µg of protein were subjected to digestion using Trypsin (1:50 – Trypsin:Protein w/w) (only human DU145 cells) or Lys-C (1:100 Lys-C:Protein w/w). After 12 h of enzymatic digestion, peptides were extracted by acidifying samples with 1% formic acid (FA) and subsequent desalting on HLB cartridges. Samples were re-suspended in 100 µL 4% FA. Samples were analyzed by LC-MS/MS using a Proxeon nanoflow HPLC system coupled to a Q-Exactive HF mass spectrometer (Thermo Fisher Scientific). Each sample was loaded and separated on a reverse-phase analytical column (18 cm length, 150 µm i.d.) (Jupiter C_{18}, 3µm, 300 Å, Phenomenex) packed manually. LC separations were performed at a flow rate of 0.6 µL/min using a linear gradient of 0-70% aqueous acetonitrile (0.2% FA) in 240 minutes. MS spectra were acquired with a resolution of 60,000. Data dependent MS/MS acquisition on the most intense precursor ions were obtained using high energy dissociation (HCD) with a frequency of up to 20 MS/MS per s. AGC target values for MS and MS/MS scans were set to 5e5 (max fill time 200 ms) and 5e4 (max fill time 200 ms), respectively. The precursor isolation window was set to m/z 1.6 with a HCD normalized collision energy of 25. The dynamic exclusion window was set to 30s.

Proteomics data analysis. Raw data analysis of SILAC experiments was performed using Maxquant software 1.5.0.8 and Andromeda search engine. False discovery rate was obtained

from concatenated database containing forward and reverse sequences was set to <1% for peptide and protein identifications. Andromeda searches were performed using the Uniprot Mouse database (Aug. 2015, 16,717 entries) and the human database (Apr. 2016, 48,130 entries). Additional data analysis was achieved using the programming language R (https://www.r-project.org/). Biomass obtained through macropinocytosis was calculated using SILAC fold ratios. Minimal biomass corresponds to the ratio of Lys-C peptides considering only mixed peaks. Maximum biomass was determined from the sum of ratios of mixed and heavy Lys-C peptides of a given protein. All ratios were calculated relative to the control (light) peak for each peptide. Only peptides containing zero missed cleavages and one arginine were used for calculation of the biomass. The minimum and maximum biomass for each peptide was determined as follows:

$$Min.\,biomass\ [\%] = \frac{0.5 * \frac{\text{Mixed Peptide}}{\text{Control}}}{0.5 * \frac{\text{Mixed Peptide}}{\text{Control}} + 1} * 100$$

$$Max.\,biomass\ [\%] = \frac{0.5 * \frac{\text{Mixed Peptide}}{\text{Control}} + \frac{\text{Heavy Peptide}}{\text{Control}}}{0.5 * \frac{\text{Mixed Peptide}}{\text{Control}} + \frac{\text{Heavy Peptide}}{\text{Control}} + 1} * 100$$

Where $\frac{\text{Mixed Peptide}}{\text{Control}} = \frac{\text{R0K4}}{\text{R0K0}} + \frac{\text{R10K0}}{\text{R0K0}}$ and $\frac{\text{Heavy Peptide}}{\text{Control}} = \frac{\text{R10K4}}{\text{R0K0}}$

All isotope ratios used are reported by MaxQuant.

MaxQuant outputs from tryptic digests of human DU145 were used to verify the Lys-C approach as follows: Proteins uniquely identified in human DU145 tryptic digests were selected relative to a database search against the mouse database. Biomass calculation for the tryptic digest of

these uniquely human proteins was performed as for maximum biomass except that mixed peptide ratios are zero due to the fact that mixed peaks are only observable upon Lys-C digestion (see equation: Max.biomass).

FLIM-FRET: Transfection and microscopy. MEFs were grown in DMEM supplemented with 10% FBS and antibiotics at 37°C in a humidified incubator in 5% CO2. Cells were transfected (calcium phosphate) with one of the following GTPase biosensors generously provided by the Hahn Laboratory at the University of North Carolina: (1) RAC1 FLARE dual-chain biosensor (CyPet-RAC1 and YPet-PBD); (2) Rac1 constitutively-active dual-chain biosensor (CyPet-RAC1-Q61L and YPet-PBD); (3) CyPet-RAC1 donor alone. Dual-chain biosensor are composed of two independent proteins (RAC1 and p21-activated kinase 1 (PAK1) binding domain, PBD) that associate upon RAC1 activation. Dual-chain FLARE biosensors exhibit a larger dynamic range and exhibit lower background activation than single-chain probes. Cells were imaged 24 h after transfection in 35-mm glass bottom dishes. Cells were imaged 48 h after transfection. Where indicated, cells were stimulated with 50 μM A769662.

FLIM-FRET measurements of the RAC1 FLARE biosensors were made on an Olympus FluoView FV1000 (Olympus, Japan) system with IX81 microscope coupled to a 2-photon excitation Ti:Sapphire laser (Spectra-Physics Mai Tai, Newport Beach, CA) producing 80 fs pulses at a repetition of 80 MHz. FILM data was acquired using an A320 Fast-FILM-box (ISS, Inc, Champaign, IL). Measurements were made using a water immersion UPL SAPO Olympus objective 60x with a NA 1.2. An SP 760 nm dichroic filter was used to separate the fluorescence signal from the laser light. The fluorescence was detected using two photomultiplier detectors (H7422P-40, Hamamatsu, Japan) and the wavelength selection by two bandpass filter (470/22 for CFP and 542/27 nm for YFP) split with a 509 nm long pass filter used as a dichroic mirror. For image acquisition, the pixel frame size was set to 256 x 256 and the pixel dwell time to 20

μs/pixel. For the time sequences, 1000 frames were acquired and then 10 frames averaged for each step. The average laser power at the sample was maintained at the mW level. FLIM data were acquired and processed using the SimFCS software package developed at the Laboratory for Fluorescence Dynamics (www.lfd.uci.edu). Calibration of the system was performed using a solution of 10 μM of Coumarin 6 in ethanol (lifetime of 2.5 ns, ISS webpage at www.iss.com).

FLIM-FRET: Phasor plot Transformation. To analyze the fluorescence decay of the RAC1 biosensor we used the phasor plot approach. Phasors plots follow simple vector algebra rules. The phasors are represented in a 2D polar plot where the fractional contribution of one or more independent lifetime fluorescent species in one pixel can be evaluated (Digman et al., 2008). In Figure S5A (black and red lines) we show an example of two different fluorescent species with different decay times. In order to implement the phasor approach the time decay at each pixel is transformed into the Fourier space that provides 2 coordinates (G,S) for each pixel, see equation 1 and 2. The universal circle in the phasor plots (semicircle line at Figure S5A); defines all the possible positions for a single exponential decay between 0 to infinite lifetime. This characteristic feature of the phasor plot is a useful tool to identify a single exponential decay without fitting or previous knowledge. When two fluorophores are mixed in a pixel, the temporal analysis should show a multi-exponential decay as represented by the green line in Figure S5A. In the phasor plot, the position for a pixel which contains multi-exponential decay should be at the line that joins the points for the two single exponential fluorophores (red and black circles). Using the additivity properties of the vectors it is possible to weigh the fractional contribution of those two fluorescent species. Finally, by using the reciprocity of the Fourier transformation it is possible to map any region of interest in the phasor plot by moving a cursor and highlighting the pixels in the image inside the cursor in the phasor plot.

In this work for each pixel in the image the fluorescence decay $I_{ij}(t)$ acquired by the FLIM card was Fourier transformed in the real and imaginary part or the so called *G* and *S* coordinates as defined by the expression:

$$\text{x-coordinate} = G_{ij(\omega)} = \frac{\int_0^\infty I_{ij}(t)\, cos(\omega t)\, \mathrm{d}t}{\int_0^\infty I_{ij}(t)_{\mathrm{d}t}} \quad (1)$$

$$\text{y-coordinate} = Sij_{(\omega)} = \frac{\int_0^\infty I_{ij}(t)\, sin(\omega t)\, \mathrm{d}t}{\int_0^\infty I_{ij}(t)_{\mathrm{d}t}} \quad (2)$$

Where, $I_{ij}(t)$ is the intensity at a given time, and ω is the laser repetition angular frequency ($2\pi f$), and the indexes *i* and *j* identify a pixel of the image.

The phasor plot transformation becomes a powerful approach to analyze the lifetime image information, avoiding fitting or complex algorithms and with a simple visual inspection. For a detailed information of phasor transformation and analysis refer to references (Digman et al., 2008; Jameson et al., 1984; Štefl et al., 2011; Weber, 1981).

FLIM-FRET: FRET-Phasor Analysis. FRET experiments can be analyzed by evaluating the fluorescence lifetime of the donor rather than the fluorescence intensity of the donor/acceptor with the benefit that FLIM measurements are independent of protein concentration. FLIM reports FRET efficiency (fraction of RAC1-GTP) in a manner that is insensitive to varying concentrations of CyPet-RAC1-GTP and YPet-PBD in a given region of the cell. Changes in FRET efficiency are readily detected by exploiting the properties of the phasor plot. In the case of a dual-chain biosensor, which has a fixed FRET efficiency, the shift of from the donor alone phasor towards the maximal FRET efficiency of the biosensor (determined using the RAC1-Q61L “GTP locked” mutant biosensor) represents the fraction of molecules with FRET (degree of RAC1 activation).

To determine the RAC1 biosensor activity (see scatter plot in Figure 2F), we apply a calculation based on weighting the pixels that have a positive FRET (*f*FRET, quenched-donor) over the total number of pixels selected (*f*FRET + *fnon*-FRET unquenched donor +quenched-donor).

$$\%\ \text{RAC1 Biosensor Activation} = \frac{fFRET}{(fFRET + fnon - FRET)} * 100$$

The selection of the pixels was done by the use of two cursors, one related with the unquenched-donor and the second one that selects all the quenched-donor. The color scale is an indicator of the effective activation of the RAC1 biosensor (red negative FRET and green positive FRET); see Figure 2F or S2B. For a more detailed description about FRET-phasor analysis refer to Hinde et al. (Hinde et al., 2012).

For the FRET movies, we used the generalized polarization function (GP analysis) to identify the population of high FRET as a change in the intensity between donor and acceptor channels, as:

$$GP = \frac{I_{Ch2} - I_{Ch1}}{I_{Ch1} + I_{Ch2}}$$

GP is a ratiometric method and can produce values between -1 to +1 that can be translated to low and high FRET, respectively.

In vivo experiments. Experiments conducted in mice were performed in accordance with the Institutional Animal Care and Use Committee of University of California, Irvine following a power analysis conducted in consultation with the Biostatistics Shared Resource of Chao Family Comprehensive Cancer Center at UCI. Prostate isografts were produced by injecting 5 million

mPCE cells subcutaneously in the flank of 25 male 5 wk old C57BL/6 mice. Once tumors reached 100 mm^3, animals were randomly placed in EIPA or vehicle group (n=10-11). Four mice injected with cells failed to grow tumors and were excluded from the study. Eleven mice were treated with 7.5 mg/kg EIPA and 10 mice were treated with 1% DMSO in PBS (vehicle) by subcutaneous injection every other day for 28 d. Tumor volume was calculated using the formula, volume (mm^3) = length [mm] x (width [mm])^2 x 0.52 as measured by calipers.

For in vivo dextran uptake analysis, JAX PDX TM00298 tumors were intratumorally injected with 2 mg Oregon Green dextran dissolved in 1% Evan's Blue Dye 1 h after intraperitoneal (i.p.) injection with vehicle or 10 mg/kg EIPA. Ten wk old *Pten*$^{flox/flox}$;*p53*$^{flox/flox}$;*PB-Cre4* male mice with prostate tumors or C57BL/6 mice bearing mPCE isografts were intravenously injected with 250 mg/kg FITC-Ficoll dissolved in 1% Evan's Blue Dye 1 h after i.p. injection of vehicle or 10 mg/kg EIPA or 1.5 h after subcutaneous injection of 7.5 mg/kg EIPA, respectively. Tumors were excised 0.5-1 h later and sections that were confirmed to blue from Evan's Blue Dye were frozen in OCT at -80 °C. Cryosectioned tumor slices were processed by the Pathology Research Services Core Facility at UC Irvine then fixed in 4% paraformaldehyde, probed for LAMP1, and evaluated on Nikon Eclipse Ti spinning disk confocal microscope. ImageJ was used to process the images and max-projection of 10-15 z-slices is shown.

Statistical methods and data analysis. Significance was determined using an unpaired t-test to compare experimental groups to controls as indicated. *, P <0.05; **, P <0.01; ***, P <0.001; n.s., not significant (P >0.05). For multiple comparisons, Tukey's method was used to determine the P-value.

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